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*Frank C. Eisenschenk*

Frank C. Eisenschenk, Ph.D., Patent Attorney

REQUEST FOR CERTIFICATE OF  
CORRECTION UNDER 37 CFR 1.322  
Docket No. G-038US03DIV  
Patent No. 6,955,902

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Ilya Chumakov, Hiroaki Tanaka  
Issued : October 18, 2005  
Patent No. : 6,955,902  
For : High Throughput DNA Sequencing Vector

Certificate  
MAR 08 2006  
of Correction

Mail Stop Certificate of Corrections Branch  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION  
UNDER 37 CFR 1.322 (OFFICE MISTAKE)

Sir:

A Certificate of Correction (in duplicate) for the above-identified patent has been prepared and is attached hereto.

In the left-hand column below is the column and line number where errors occurred in the patent. In the right-hand column is the page and line number in the application where the correct information appears.

**Patent Reads:**

Title page (\*) Notice, line 3:

“U.S.C. 154(b) by 402 days.”

**Application Reads:**

Terminal Disclaimer filed February 23, 2004:

-- U.S.C. 154(b) by 402 days.

This patent is subject to a terminal disclaimer.--

Column 18, line 16:

“stra+ gene”

Column 19, line 36:

“lacZ”

Column 26, line 49:

“calorimetrically”

Column 31, line 46:

“stra+ gene”

Column 33, line 8:

“20 µl kanamycin”

Column 34, line 21:

“pGenDel 1 pXRD4043”

Column 49, line 30:

“EcoRI site”

Page 26, line 5:

--strA+ gene--

Page 28, line 3:

--lacZ--

Page 38, line 26:

--colorimetrically--

Page 45, line 30:

--strA+ gene--

Page 47, lines 30-31:

--20µg/ml kanamycin--

Page 49, line 22:

--pGenDel1. pXRD4043--

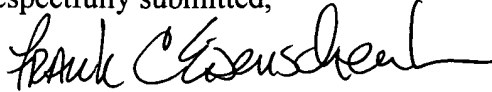
Page 71, line 18:

--EcoRI site--.

A true and correct copy of Terminal Disclaimer filed February 23, 2004 and pages 26, 28, 38, 45, 47, 49, and 71 of the specification as filed which support Applicants' assertion of the errors on the part of the Patent Office accompanies this Certificate of Correction.

Approval of the Certificate of Correction is respectfully requested.

Respectfully submitted,



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FCE/gyl

Attachments: Copy of pages 26, 28, 38, 45, 47, 49, and 71 of the specification  
Copy of Terminal Disclaimer filed February 23, 2004



In some embodiments, the vector may contain more than one copy number indicator. For example, as shown in Figure 1 and SEQ ID NO:1, pGenDel1 contains two copy number indicators. One of the copy number indicators is the rpsL gene (GenBank Accession number J01688, the disclosure of which is incorporated herein by reference), which is also referred to as the strA<sup>+</sup> gene. In pGenDel1, the strA<sup>+</sup> gene is located between base 6574 and base 7218 of SEQ ID NO:1, between the HpaI end of the pBeloBAC11 fragment and the left end of Tn1000. However, those skilled in the art will appreciate that the copy number indicator is not limited to this location and may be located at any position in the vector consistent with its intended purpose. Similarly, it will be appreciated that the copy number indicator may be any copy number indicator consistent with its intended purpose, and that the copy number indicator is not limited to those specifically listed herein.

The strA<sup>+</sup> gene in pGenDel1 has been modified to change the base corresponding to position 342 of the strA<sup>+</sup> gene of GenBank Accession number J01688 (position 7058 in pGenDel1) from A to T. This modification does not affect the amino acid sequence of the translated protein but removes a PmlI site in order to maintain the PmlI site in the high copy number origin of replication as a unique site. The present vectors utilize the new observation that when the strA<sup>+</sup> gene is present in high copy number, it confers streptomycin sensitivity on cells which are normally streptomycin resistant. Thus, prior to cloning of an insert into the high copy number origin of replication, host cells which are normally resistant to streptomycin will be sensitive to streptomycin as a consequence of the high copy number of the strA<sup>+</sup> gene. When an insert is cloned into the high copy number origin of replication thereby reducing the copy number of the vector, the cells will become resistant to streptomycin. Thus, streptomycin resistance may be used to positively select cells containing vectors with inserts in the high copy number origin of replication.

In addition to the strA<sup>+</sup> gene, pGenDel1 contains a second marker indicative of the copy number of the vector in the host cell. This second marker is a truncated version of the LacZ gene which was obtained as follows. The double stranded synthetic linker HE1 having the sequence AGCTACGGGAAAGCC (SEQ ID NO:4)/HE2:AATTGGCTTTCCCGT (SEQ ID NO: 20) was ligated to pUC19 DNA which had been digested with HindIII and EcoRI, thereby eliminating the HindIII and EcoRI sites in pUC19. When transformed into bacterial host cells, the modified pUC19 plasmid gives

be used as a deletion indicator which allows identification or selection of cells in which transposition has generated a deletion within the insert in the high copy number origin of replication. The use of the truncated lacZ gene for this purpose is described below.

#### D. Vector Maintenance Marker

5       The vectors of the present invention also include a marker for selecting host cells which contain the vector. This selectable marker may be any of a variety of selectable markers known to those skilled in the art. In particular, the selectable marker may confer resistance to a drug, biomolecule, metabolite, or other agent.

10       In pGenDel1, the marker for selecting host cells harboring the vector is the kan<sup>r</sup> gene derived from bases 720 through 1535 of the plasmid pUC4K (GenBank Accession No. X06404, the disclosure of which is incorporated herein by reference) available from Pharmacia. The kan<sup>r</sup> gene is located between bases 9 and 1266 of pGenDel1. However, those skilled in the art will appreciate that the marker for selecting host cells harboring the vector is not limited to this location or this particular marker. Instead, the marker for  
15       selecting host cells harboring the vector may be any marker consistent with its intended purpose and may be located at any position in the vector consistent with its intended purpose.

20       In the pGenBac vectors, the vector maintenance marker is the chloramphenicol acetyl transferase gene, which confers resistance to chloramphenicol. Those skilled in the art will appreciate that a number of other plasmid maintenance markers may also be used. The present invention contemplates the use of any plasmid maintenance marker consistent with the purposes described herein.

#### E. Insert Amplification Primers

25       In a preferred embodiment, hybridization sites for insert amplification primers are located on each side of the cloning sites in the high copy number origin of replication. The insert amplification primers may have any sequence complementary to a sequence near the cloning sites in the high copy number origin of replication. Preferably, the insert amplification primers are at least 15 nucleotides in length. More preferably, the insert amplification primers are between 15 and 25 nucleotides in length.

30       The hybridization sites for insert amplification primers may be from about 20 to about 800 bases from the cloning sites in the high copy number origin of replication. Preferably, the hybridization sites for insert amplification primers are from about 20 to

## VECTORS DESIGNED FOR GENERATING NESTED

### DELETIONS USING ENZYMATIC METHODS

5 The features of high throughput sequencing vectors which are enzymatic deletion vectors are described below. Each of the vectors for generating nested deletions using enzymatic methods has the features listed below. While pGenDel1 is used as a representative of this class of vectors, it will be appreciated that this class of vectors need not include all the features of pGenDel. Instead, this class of vectors comprises the features listed below.

10 The vectors for generating nested deletions using enzymatic methods contain a high copy number origin of replication having cloning sites therein, a low copy number origin of replication and any controlling genes required for the activity of the low copy number origin of replication, a deletion indicator and at least one copy number indicator as described above. The enzymatic deletion vectors may also have hybridization sites for primers for creating substrates for enzymatic deletions, hybridization sites for primers for  
15 amplifying deletions, and hybridization sites for primers for sequencing deletions. In addition, deletion vectors may also have the other features described above, such as a vector maintenance marker, hybridization sites for insert amplification primers, hybridization sites for insert sequencing primers, and a single stranded origin of replication.

#### 20 A. Deletion Indicator

Preferably, the vectors designed for use in enzymatic methods for generating nested deletions have at least one deletion indicator for indicating when an enzymatic deletion has progressed into the insert in the high copy number origin of replication. The deletion indicator is preferably a marker for which the loss thereof through enzymatic  
25 deletion is readily detectable. The marker may be a selectable marker or a readily assayable protein, such as a colorimetrically detectable protein such as beta galactosidase. The deletion indicator is preferably of short length to minimize the recovery of deletions which have deleted enough of the deletion indicator to destroy the activity of the deletion indicator but have not progressed into the insert in the high copy number origin of  
30 replication. In those embodiments which are designed for generating nested deletions using both enzymatic and transposition-based methods, the deletion indicator for determining whether an enzymatic deletion has proceeded into the insert may also act as a

copy number replication. The high copy number origin of replication facilitates the preparation and manipulation of vector DNA, a crucial feature which limits the applicability of the current oriS containing plasmids.

5 Cells carrying the pGenDel1 vector without an insert are kanamycin resistant, streptomycin sensitive, sucrose sensitive, and dark blue on Xgal and IPTG. As described in more detail below, cells carrying pGenDel1 derivatives in which inserts have been cloned into the cloning sites in the high copy number origin of replication are kanamycin resistant, streptomycin resistant (where streptomycin resistant host cells are used), sucrose sensitive, and light blue on Xgal and IPTG. Moreover, aberrant plasmids resulting from  
10 rearrangement or degradation during the cloning steps usually lack an intact lacZ gene and are easily identified due to their white coloration in the presence of IPTG/Xgal.

Upon cloning an insert into the cloning sites in the high copy number origin of replication, the function of the high copy number origin of replication is disrupted and replication of the vector is directed by the low copy number origin of replication. In  
15 addition to simplifying the generation and sequencing of nested deletions using transposition based methods or enzymatic methods as discussed below, the low copy number of the resulting recombinants allows the stable cloning of fragments from several hundreds of bases to several hundreds of kilobases in length and permits the maintenance of sequences which are unclonable in high copy number.

20 In pGenDel1, the unique PmlI and ScaI blunt ended cloning sites in the high copy number origin of replication (ori pUC19 derived), allow the cloning of inserts at these sites, which in turn provokes a drastic change in the number of molecules per cell. The strA<sup>+</sup> gene carried by pGenDel1 renders streptomycin-resistant recipient cells sensitive to streptomycin when present at a high copy number. Thus, streptomycin-resistant cells  
25 transformed by a pGenDel1 plasmid without an insert in the high copy number origin of replication (ori pUC19), will be sensitive to streptomycin and unable to grow on streptomycin-containing media. However, cells containing recombinant pGenDel1 plasmids with inserts in the high copy number origin of replication (ori pUC19) will be present at low copy numbers and will be able to grow in the presence of streptomycin.

30 Cells containing rare spontaneous mutations which inactivate the strA<sup>+</sup> gene would also grow on streptomycin. However, such cells can be effectively detected and rejected using the truncated LacZ color-based selection system. Cells containing

The resulting fractions of 4 -6 kb size were excised and DNA was extracted by electroelution and subsequent concentration on Microcon 100 columns.

The ends of the resulting DNA fragments were repaired by treatment with 4 units of Vent DNA polymerase (New England Biolabs) in the presence of 125  $\mu$ M of all four dNTPs, for 20 minutes at 72°C in a final volume of 20  $\mu$ l of ThermoPol buffer supplied by the manufacturer. The DNA was extracted with chloroform, precipitated in ethanol and resuspended in 10mM Tris-HCL pH 7.5, 1 mM EDTA. DNA concentrations were estimated by relative fluorescence of agarose gels containing lambda phage DNA samples of known concentrations.

#### 10           B. Preparation of Vector DNA

pGenDell vector DNA was extracted from overnight cultures by the standard alkaline lysis procedure. In order to check the integrity of the vector, 1 $\mu$ l of the overnight cultures was plated on LB agar containing either sucrose and kanamycin or streptomycin and kanamycin. Only cultures giving no more than 5-10 resistant colonies in these tests were used for further DNA extraction. Supercoiled plasmid was obtained by Ethidium bromide/CsCl gradient centrifugation. 2 $\mu$ g of the resulting plasmid DNA was then digested with 10 units of ScaI enzyme, in conditions recommended by the manufacturer (New England Biolabs). After adding EDTA at a 10mM concentration, the vector DNA was extracted with phenol, and concentrated by Microcon 100 centrifugation.

#### 20           C. Ligation of Insert DNA into Vector and Selection of Clones Having Inserts

10ng of pGenDell vector DNA was then ligated in a 10 $\mu$ l reaction volume, with 50ng of the BAC fragment DNA, in the presence of 200 units of T4 DNA ligase (Epicentre) according to manufacturer's recommendations, at 12°C overnight. Following addition of 20 $\mu$ g of bacterial tRNA, the ligation products were extracted with phenol and precipitated in ethanol.

D10HB cells containing or lacking the transposase expression plasmid pXRD4043 were washed, and subjected to electroporation in the presence of the purified ligation products, in a 40 $\mu$ l final reaction volume. Following the addition of L broth, the mixtures were incubated for 45 minutes at 30°C (to reduce transposition), and plated in 20 $\mu$ l aliquots on the surface of 100 mm Petri dishes containing 2% agar-LB, 20 $\mu$ g/ml kanamycin, 20 $\mu$ g/ml chloramphenicol (in procedures using DH10B cells containing pXRD4043, which confers chloramphenicol resistance), 100 $\mu$ g/ml streptomycin, IPTG

DH10B cells lacking the pXRD4043 plasmid, out of 36 colonies tested, 28 gave inserts of 4-6 kb, 3 contained inserts of less than 600bp and 5 failed to amplify any product.

For DH10B cells containing the pXRD4043 plasmid, out of 192 colonies tested 19 had inserts of less than 400bp in length, 26 had inserts between 400bp and 1kb in length, 5  
5 had inserts between 1kb and 5kb in length, 130 had inserts between 4 and 7kb in length, and 12 gave no amplification product.

On average, 90%-95% of the colonies contained an insert and 70-80% of the colonies contained inserts in the expected size range of 4-7kb.

The ends of the inserts present in the PCR products were sequenced on ABI  
10 automatic sequencers using the OS1 and OR1 primers.

The cloning and selection of pGenDel1 recombinants having inserts in the high copy number origin of replication is summarized in the section of Figure 2B in which the insert is cloned into pGenDel1. As indicated in Figure 2B, cells containing pGenDel1 derivatives containing an insert are kanamycin resistant, streptomycin resistant, sucrose  
15 sensitive and faint blue on IPTG/Xgal plates. Thus, such cells can be selected and identified on IPTG/Xgal plates containing kanamycin and streptomycin.

## II. Generation and Analysis of Nested Deletions Using Transposition Based Methods

For the generation of nested deletions by transposition, pGenDel1 is introduced into cells containing a source of transposase. As described above, the bacterial strain  
20 D10HB, containing the plasmid pXRD4043 (Tsai, M.-M., Wong, R.R., Hoang, A.T., Deonier, R.C. (1987) J. Bacteriol. 169, 5556-5562.) was used for the initial cloning of inserts into pGenDel1. pXRD4043 is a moderate copy number pACYC184 plasmid which contains the *tnpA* gene under the control of a synthetic *tac* promoter. As a selectable marker, pXRD4043 contains the chloramphenicol resistance gene. However, it will be  
25 appreciated that the selectable marker on the plasmid which serves as a source of transposase may be any of a variety of selectable markers, provided that the same selectable marker is not on the vector containing the insert in which deletions are to be generated.

Initially, the cells are grown at 30°C in order to restrict transposition. When cells  
30 are cultivated at 37°C, the transposase becomes active and mediates the intramolecular transposition of the Tn1000 ends within pGenDel1. Transposition based deletions which result in linking the left transposon end to any position within the cloned insert will remove

sequences which may be toxic if present at a high copy number. These features may be particularly advantageous when constructing genomic DNA libraries such as BAC libraries.

5 Vectors useful in such applications comprise a high copy number origin of replication having at least one cloning site therein which is positioned such that the ability of the high copy number origin of replication to direct replication is lost when an insert is cloned into the cloning site. In addition, the vectors comprise a low copy number origin of replication and any genes necessary for the activity of the low copy number origin of replication, at least one copy number indicator for indicating the copy number of the vector  
10 in the host cells, and a vector maintenance marker for selecting cells containing the vector. If desired, the vectors may also include hybridization sites for primers for amplifying the insert and hybridization sites for primers for sequencing the amplified insert.

Exemplary vectors for maintaining inserts at low copy number (designated pGenBac1 and pGenBac2) are shown in Figures 13 and 14. Each of these vectors contain  
15 the high copy number origin of replication from pUC19 with a unique cloning site therein. In pGenBac1 the unique cloning site in the high copy number origin of replication is an EcoRI site, while in pGenBac2 the unique cloning site in the high copy number origin of replication is a BamHI site. When an insert is cloned into the EcoRI site of pGenBac1 or the BamHI site of pGenBac2, the activity of the high copy number origin of replication is  
20 disrupted. However, as described above, vectors having inserts therein are capable of replicating at low copy number under the control of oriS and its associated regulatory genes.

pGenBac1 and pGenBac2 also contain the strA gene which permits the selection of streptomycin resistant host cells carrying vectors having inserts therein as described above.  
25 In addition, pGenBac1 and pGenBac 2 contain the truncated lacZ gene described above as a copy number indicator.

A gene conferring resistance to chloramphenicol is used as a vector maintenance marker. In addition, pGenBac1 and pGenBac2 also contain hybridization sites for the oriLRd and oriLRr PCR primers described above to permit amplification of the insert  
30 DNA. Hybridization sites for the OS1 and OR1 sequencing primers are also included to allow the amplified insert to be sequenced.

Example 7 describes the construction of pGenBac1 and pGenBac2.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,955,902

Page 1 of 1

APPLICATION NO.: 09/849,866

DATED : October 18, 2005

INVENTORS : Ilya Chumakov, Hiroaki Tanaka

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page:

No. (\*), "U.S.C. 154(b) by 402 days." should read

-- U.S.C. 154(b) by 402 days.

This patent is subject to a terminal disclaimer.--.

Column 18,

Line 16, "stra+ gene" should read --strA+ gene--.

Column 19,

Line 36, "lacz" should read --lacZ--.

Column 26,

Line 49, "calorimetrically" should read --colorimetrically--.

Column 31,

Line 46, "stra+ gene" should read --strA+ gene--.

Column 33,

Line 8, "20µl kanamycin" should read --20µg/ml kanamycin--.

Column 34,

Line 21, "pGenDel 1 pXRD4043" should read --pGenDel1. pXRD4043--.

Column 49,

Line 30, "EcoRi site" should read --EcoRI site--.

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UNITED STATES PATENT AND TRADEMARK OFFICE

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Docket No. G-038US03DIV  
Serial No. 09/849,866

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner : Jane J. Zara  
Art Unit : 1635  
Applicants : Ilya Chumakov, Hiroaki Tanaka  
Serial No. : 09/849,866  
Filed : May 4, 2001  
Conf. No. : 1458  
For : High Throughput DNA Sequencing Vector

Commissioner for Patents  
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TERMINAL DISCLAIMER

Sir:

The owner, Genset S.A., of 100% interest in the above-identified patent application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application, which would extend beyond the expiration date of the full statutory term defined in 35 U.S.C. 154 to 156 and 173, as shortened by any terminal disclaimer, of prior Patent No. 6,022,716. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 to 156 and 173 of the prior patent, as shortened by any terminal disclaimer, in the event that it later expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination

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certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer.

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☒ I authorize the Patent Office to charge the amount of ☐ \$55.00 (small entity)  
☒ \$110.00 (large entity) for the terminal disclaimer fee under 37 CFR 1.20(d) to Deposit  
Account No. 19-0065.

February 23, 2004  
Date

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